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2018-04

Shah , F H , Mali , T L E & Lundell , T K 2018 , ' Polyporales brown rot species Fomitopsis pinicola : enzyme activity profiles, oxalic acid production, and Fe3+-reducing metabolite secretion ' , Applied and Environmental Microbiology , vol. 84 , no. 8 , UNSP e02662-17 . https://doi.org/10.1128/AEM

http://hdl.handle.net/10138/307257
https://doi.org/10.1128/AEM.02662-17

acceptedVersion

Polyporales brown rot species *Fomitopsis pinicola*: enzyme activity profiles, oxalic acid production, and Fe^{3+}-reducing metabolite secretion

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Running title: Biochemical activity profile of brown rot *F. pinicola*

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Abstract

Basidiomycota fungi in the order Polyporales are specified to decomposition of dead wood and woody debris, and thereby, are crucial players in degradation of organic matter and cycling of carbon in the forest ecosystems. Polyporales wood-decaying species comprise of both white-rot and brown-rot fungi, based on their mode of wood decay. While the white-rot fungi are able to attack and decompose all the lignocellulose biopolymers, the brown-rot species mainly cause destruction of wood polysaccharides with minor modification of the lignin units. The biochemical mechanism of brown-rot decay of wood is still unclear, and has been proposed to include a combination of non-enzymatic oxidation reactions and carbohydrate-active enzymes. Therefore, a linking approach is needed to dissect the fungal brown-rot processes. We studied the brown-rot species *Fomitopsis pinicola*, by following mycelial growth and enzyme activity patterns, and generation of metabolites together with Fenton promoting Fe^{3+}-reducing activity, for three months in submerged cultures and supplemented with spruce wood. Enzyme activities to degrade hemicellulose, cellulose, proteins and chitin were produced by three isolates of *F. pinicola*. Substantial secretion of oxalic acid and decrease in pH were notable. Aromatic compounds and metabolites were observed to accumulate in the fungal cultures, with some metabolites having Fe^{3+}-reducing activity. Thus, the brown-rot species *F. pinicola* demonstrates a pattern of strong mycelial growth leading to active production of carbohydrate and protein-active enzymes, together with promotion of Fenton biochemistry. Our findings point to fungal species-level “fine-tuning” and variations in the biochemical reactions leading to brown-rot type of decay of wood.

Importance

Fomitopsis pinicola is a common fungal species in boreal and temperate forests in the northern hemisphere encountered as a wood-colonizing saprotroph and tree pathogen, causing a severe brown-rot type of degradation of wood. However, its lignocellulose-decomposing mechanisms have remained undiscovered. Our approach was to explore both the enzymatic activities, and non-enzymatic Fenton reaction promoting activities (Fe^{3+}-reduction, metabolite production), by cultivating three isolates of *F. pinicola* in lignocellulose-supplemented cultures. Our findings on the simultaneous production of versatile enzyme activities, including endoglucanase, xylanase, β-glucosidase, chitinase, and acid peptidase, together with generation of low pH, accumulation of oxalic acid and Fe^{3+} reducing metabolites, open up the variations of fungal brown-rot decay mechanisms. Furthermore, these findings will aid us in revealing the wood-decay proteome, transcriptome and metabolic activities of this ecologically important forest fungal species.

Introduction

The order Polyporales is a diverse group of fungi belonging to the Agaricomycetes, containing many efficient wood-decay species which are capable of degrading lignocellulose (1). The Polyporales are mainly saprotrophic wood-decay fungi, including a few species of plant pathogens (2). Two major wood-decaying types are classified within the Polyporales: white-rot fungi that are able to attack and modify aromatic lignin units as well as the polysaccharide-carbohydrate polymeric compounds of wood, and brown-rot fungi that decompose wood

64 cellulose and hemicelluloses mainly by a combinations of non-enzymatic oxidation *via* Fenton
65 chemistry, and carbohydrate-active enzyme (CAZy) activities (3-9).

66 In this study, we describe enzymatic and biochemical activities generated by the brown-rot
67 Basidiomycota species *Fomitopsis pinicola* (Swartz: Fr.) Karst., a bracket-forming fungus
68 classified in the order Polyporales of the systematic class Agaricomycetes. The species has a
69 worldwide distribution around temperate and boreal forests, being also abundant in the
70 forests of Finland and Northern Scandinavia, and causes distinctive brown-colored wood-
71 decay in conifers and hardwoods (10). The sequenced and annotated genome of *F. pinicola*
72 demonstrates the presence of a set of genes encoding CAZys and auxiliary oxidoreductases
73 (6), thereby suggesting their role in the lignocellulose-degrading system of the fungus.
74 However, production of wood-decay enzyme activities in connection to the brown-rot
75 promoting Fenton reactions have not been studied for the species before.

76 In brown-rot fungi, the proposed two-step process of wood-decay involves hydroxyl radical-
77 mediated oxidation of lignocellulose *via* Fenton reaction, followed by the enzymatic
78 degradation of cellulose and hemicellulose (9, 11-14). The generation of hydroxyl radical *via*
79 Fenton reaction requires active reduction of Fe^{3+} ions to Fe^{2+}, acidic conditions, and supply of
80 hydrogen peroxide (14). The brown-rot oxidation process and generation of hydroxyl radicals
81 has been proposed to be spatially and/or temporally segregated from hydrolytic enzymes *in*
82 *order* to limit deactivation by hydroxyl radicals (15-16).

83 Brown-rot fungi secrete oxalic acid in significant quantities, chelating Fe^{3+} in the vicinity of
84 hyphae at low pH (<3), which restrains reduction of Fe^{3+} and initiation of Fenton chemistry
85 (15-17). Thus, the low pH micro-environment protects the fungal hyphae against oxidation by
86 hydroxyl radicals. Oxalic acid concentration is proposed to decrease gradually away from the

hyphae by diffusion, leading to increasing pH at the wood matrix and xylem cell walls (pH >5), thus resulting in dissociation of the Fe^{3+}-oxalate chelates. Low molecular weight reductants (metabolites) secreted by the fungus or compounds released from the woody matrix could then generate Fe^{2+} from the Fe^{3+} chelates, and thereby fueling Fenton reaction and subsequent oxidative attack leading to opening up of the lignocellulose matrix (9, 12, 15, 17).

The Fenton-based oxidative system has been proposed to break down the lignocellulose matrix in wood, loosening up the xylem cell walls, and thereby exposing the composite fine structure of cellulose microfibrils and covering hemicellulose and lignin components, to the lignocellulose-attacking CAZy enzymes secreted by the fungus (9). Comparative genomic analyses and evolutionary studies of wood-decaying Agaricomycetes and Polyporales fungi have made it easier to understand the genetic and biological factors directing white- or brown-rot decay mechanisms (1, 5-7,18). Hemicellulose- and cellulose-active enzymes have previously been studied in a few species of brown-rot fungi, suggesting that hemicellulose may be decomposed prior to cellulose degradation (19-22). Xylanases, endoglucanases and glucosidases form a major part of the brown-rot enzyme complex, targeted to these components of wood lignocellulose and resulting saccharides (4, 18, 23).

Uncovering the brown-rot wood decay mechanisms using sophisticated biochemical testing and biomimetics (9, 13-16, 24), or by genomic studies including extensive sequencing and proteomics (1, 5-6, 18) has turned out to be more complicated than expected. Thus, different and more combinatory approaches are needed. In our study, three Finnish isolates of *F. pinicola* presented notable CAZy enzyme activities for decomposition of hemicellulose and cellulose, as well as for chitin and protein degradation, together with release of Fe^{3+}-reducing activity, and substantial accumulation of oxalic acid leading to increase in extracellular acidity.

110 Moreover, a few fungal-generated extracellular compounds were observed to demonstrate
111 Fe^{3+}-reducing activity, thus being potential candidates for more detailed studies on wood-
112 active metabolites and oxidative reactions in lignocellulose bioconversions.

113 **Materials and Methods**

114 *Fungal isolates*

115 Three wild-type isolates of the species *Fomitopis pinicola* (FBCC1181, FBCC1468,
116 FBCC1243) (abbreviated as Fp1181, Fp1468 and Fp1243, respectively) were obtained as
117 living hyphal stored cultures from the University of Helsinki Microbiology - Fungal
118 Biotechnology Culture Collection (HAMBI-FBCC, fbcc@helsinki.fi). Identity and taxonomy of
119 the isolates were confirmed by ITS-PCR and Blastn analyses on the ITS1-5.8S-ITS2 contig
120 sequences (25) resulting with Blast 0.0 E-value and 99-100% sequence identities with other
121 isolates of *F. pinicola*, and are deposited in ENA nucleotide sequence data bank
122 (http://www.ebi.ac.uk/ena) under accessions [LT844580] (Fp1181), [LT906565] (Fp1243), and
123 [LT906564] (Fp1468). The fungal isolates were maintained on 2 % (w/v) malt extract (Biokar
124 Diagnostics, France) agar plates at 25˚C and in the dark.

125 *Culture conditions and media*

126 For enzyme activities and metabolite production, the isolates were cultivated as previously
127 described (26). Culture media adopted for the enzyme activities and metabolite production
128 were 1 % (w/v) malt extract (Biokar Diagnostics, France) liquid (ME), pH 5.5, and low-nitrogen
129 asparagine-succinate medium, pH 4.5, containing 1 % (dw/v) of spruce (*Picea abies*) sawdust
130 wood (LNAS-wood). Carbon source in the media were either dissolved carbohydrates (ME) or
131 ground spruce wood (LNAS-wood). The media were autoclaved (121 °C, 15 min) before use.

132 Each *F. pinicola* isolate was pre-cultivated in 100 ml of ME broth for one week at 25 °C in the
133 dark and non-agitated. The mycelia were homogenized in the broth using a sterilized steel
134 container using a Waring blender. Aliquots of 2 ml of the mycelial homogenate were adopted
135 to inoculate 250 ml flasks containing 100 ml of autoclaved ME broth or wood-LNAS medium,
136 and closed with sterilized spongy stoppers. Each fungal isolate was cultivated in five parallel
137 flasks, on both media, and incubated at 25˚C in the dark as stationary cultures over periods of
138 twelve weeks of cultivation. Sample volumes of 1.5 ml for enzyme activity measurements
139 were aseptically taken from the culture fluids at time points 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10
140 weeks. Control media flasks without fungi were incubated similarly. Metabolite profiles and
141 analysis of Fe^{3+}-reduction were also followed up to 12 weeks of cultivation.

142 *Enzyme activities*

143 Lignocellulose-attacking enzyme activities produced in five parallel culture flasks of each
144 isolate on the wood-LNAS medium were measured by using 96-well plate assays and Infinite
145 M200 microplate reader spectrophotometer (Tecan, Switzerland). Reaction volume was 250
146 µl, and three parallel reactions (technical replicates) were measured for each sample from
147 each fungal culture flask. Cellulolytic endo-β-1,4-glucanase (EC 3.2.1.4) (endoglucanase) and
148 β-glucosidase (EC 3.2.1.21) activity reactions were performed in 50 mM sodium-citrate buffer
149 (pH 5) at 45°C (27). Annotated genome of *F. pinicola* indicates the absence of CAZy family
150 GH6 or GH7 cellobiohydrolase (CBH, exocellulose) encoding genes (6). Thus, specific CBH
151 activity was not assayed in this study. Endo-β-1,4-glucanase activity was determined with 1%
152 (w/v) hydroxyethyl cellulose (Sigma Aldrich) as a substrate (27). Endo-β-1,4-xylanase (EC
153 3.2.1.8) activity was assayed with birch xylan (Sigma Aldrich) as the substrate, and reducing
154 sugars were measured with dinitrosalisylic acid (DNS) at 540 nm (28). β-glucosidase activity

was assayed by quantifying *p*-nitrophenol released from 1 mM 4-nitrophenyl-β-D-glucopyranoside (Sigma Aldrich) at 400 nm (27). Solutions of varying concentrations of *p*-nitrophenol were prepared as references for quantitation of product formation in the enzyme kinetic assays. Trials for laccase activity assay using ABTS as substrate (25, 26) were performed for all three isolates on both media.

Extracellular proteolytic activity was measured using a modified version of the previously adopted method (29). The conjugate fluorescein-isothiocyanate coupled with bovine-serum albumin (FITC-BSA; Sigma Aldrich) was used as a substrate. Briefly, 100 µl of the sample was mixed with an equal volume of buffer (10 mM citrate-HCl, pH 4.0) and 10 µl of FITC-BSA (1 mg ml^{-1} in citrate buffer), and the mixture was incubated at 37˚C for 4 hours. The reaction was terminated by adding 100 µl of 10 % (w/v) trichloroacetic acid. After 1 h of incubation at room temperature in the dark, the sample was centrifuged at 16 000 *g* for 5 min at room temperature. Then, 5 µl of the supernatant was mixed with 200 µl 0.4 M boric acid-NaOH buffer (pH 8.7) in a 96-well plate. Fluorescence was measured using a spectrofluorometer (Victor, Perkin-Elmer) with excitation set to 495 nm and emission set to 520 nm. One fluorescence unit corresponds to 6.4 ng ml^{-1} of pepsin (Sigma Aldrich) incubated in the citrate-HCl buffer (pH 4.0) under the same conditions at 37˚C for 4 hours.

Extracellular chitinase activity was estimated using a method adopted for fungi (30). MU-N-acetyl-β-D-glucosaminide (Sigma Aldrich) was used as a substrate, in 96-well plates. Release of the product, 4-methylumbelliferone, was measured using the Victor spectrofluorometer (Perkin-Elmer) at excitation wavelength of 360 nm and emission at 450 nm. Calibration solutions of 4-methylumbelliferone (Sigma Aldrich) were prepared for product quantitation and enzyme activity calculations.

178 *Oxalic acid analysis*

179 Oxalic acid was chromatographically analyzed with Agilent 1290 Infinity Binary LC 171
180 System (Agilent Technologies) coupled with a guard cartridge (Agilent Technologies) and
181 Luna C_{18} column (150 mm x 4.6 mm, 3 µm particle size, Phenomenex). Column temperature
182 was 40°C, and separation was conducted at a flow rate of 0.950 ml min^{-1} under isocratic
183 conditions by using an eluent mixture consisting of 95 % (v/v) of 0.3% H_3PO_4 and 5 % (v/v)
184 acetonitrile (31). Samples were filtered using 0.2 µm pore size membrane filters (Chromacol
185 RC, Thermo Scientific) before injection (2 µl). Chromatographic separation was recorded with
186 Agilent diode-array detector and elution of oxalic acid was followed with detection of
187 absorbance at 210 nm and reference at 450 nm. Varying concentrations of high-purity grade
188 oxalic acid (Sigma Aldrich) were adopted to quantitate oxalic acid in the samples using
189 external standard method.

190 *Ergosterol analysis*

191 Total ergosterol was used to determine fungal growth and production of mycelial biomass in
192 the flask cultures. Ergosterol was extracted from wood-LNAS and ME liquid cultures by
193 freezing the wood meal and mycelium with liquid N_2, and then homogenizing with IKA
194 Analytical mill or using mortar and pestle , respectively, at cultivation time points of 0, 4, 6, 8
195 and 12 weeks, for all isolates. Ergosterol was extracted using established methods (32-33)
196 using three technical sub-samples of 100-200 mg (raw weight) from each fungal replicate
197 culture for all isolates, and measured by Agilent 1100 HPLC (Agilent Technologies), using
198 previously described elution parameters and separating column with detection of absorbance
199 at 282 nm (32). Set of ergosterol (Sigma Aldrich) concentrations were used as reference.

Fe^{3+}-reduction assay

Fe^{3+}-reducing activities were analyzed using a modified method of ferrozine assay (34). A 50-µl aliquot of the sample was mixed with 1.0 ml of 0.1 M acetate buffer (pH 4.5), 25 µl of freshly prepared 1.0 mM $FeCl_3$, and 20 µl of 1% (w/v) aqueous ferrozine (Sigma Aldrich). The reaction mixture was incubated for 5 min in the dark at room temperature. Fe^{3+}-reduction was assayed spectrophotometrically at 562 nm. A standard curve was constructed using varying concentrations of $FeSO_4$ (0.0 to 3.0 mM).

Extracellular metabolite profiling

Extracellular metabolites were extracted from the culture fluids by ethyl acetate extraction (34). Equal volumes of culture fluid filtrates obtained from the parallel culture flasks, and ethyl acetate were mixed and vortexed, with addition of 1 ml of 1 M HCl. The ethyl acetate phase was recovered and dried under a stream of N_2. The dried ethyl acetate phase was dissolved in methanol, and analyzed by using the Agilent 1290 Infinity UHPLC (Agilent Technologies) coupled with a guard cartridge and Kinetex C18 column (150 x 2.1 mm, 2.6 µm particle size, Phenomenex). A step-wise elution gradient consisting of 0.1% (v/v) formic acid in HPLC-grade water (solvent A) and 100% acetonitrile (solvent B) was applied at a flow rate of 0.36 ml/min, using step-wise gradient elution (initial hold 3 min with 95% solvent A and 5% of solvent B, followed by a linear increase in 21 min to 70% of solvent B, then in 6 min to 95% of solvent B, kept stable for further 3 min, and returning to initial condition of 5 % solvent B within 5 minutes). Elution was recorded using the Agilent diode-array detector and chromatograms were processed at 210, 260 and 280 nm wavelengths with peak-actuated spectral scanning from 200 to 400 nm. Eluted fractions of fungal-produced organic compounds were collected using the Infinity UHPLC based on their elution time and peak

height, and dried under N_2. Media compounds were eliminated from the HPLC analyses according to comparison and subtraction from the fungal culture profiles. The concentrated fractions were further analyzed for their potential for Fe^{3+}-reducing activity.

Results

Carbohydrate-active enzyme production profiles

The three isolates of *F. pinicola* exhibited hemicellulose and cellulose-degrading CAZy activities on the wood-LNAS medium, and activities of endo-β-1,4-xylanase (xylanase), endo-β-1,4-glucanase (EG), and β-glucosidase (BG) were detected (Fig 1). Extracellular laccase or peroxidase activities were not detected in the culture fluid samples (data not shown) – which is in accordance with our recent study on the Fp1181 isolate enzyme activity profile on similar media and cultivation conditions (25).

All three *F. pinicola* isolates demonstrated xylanase activity peaking within the first week of cultivation on wood-LNAS, which was then followed by a decline (Fig 1a). After four weeks, however, dissimilarities were observed in xylanase activity profiles between the three isolates. While the isolate Fp1243 demonstrated the highest xylanase activity of 8.14 µkat/L observed around cultivation week 9, the two other isolates showed very low activity levels (Fig 1a). EG activity was also detected with all isolates on wood-LNAS medium, with a fluctuating pattern (Fig 1b). Low levels of BG activity were observed with all isolates on wood-LNAS, with activities peaking either on the third or fourth week of cultivation (Fig 1c). A potential second phase for BG activity was observed with two isolates during cultivation weeks 7 to 9.

Proteolytic and chitinolytic activities

When cultivated on either ME liquid or wood-LNAS media, all three isolates of *F. pinicola* produced extracellular acidic peptidase activities with increasing levels of activity over the cultivation period of ten weeks (Fig 2). In general, higher proteolytic activities were observed on ME medium compared to wood-LNAS. On wood-LNAS, the highest peaks of activities were detected on cultivation week 8 (for Fp1468 and Fp1243) (Fig 2a). On ME medium, the isolates Fp1181 and Fp1243 in turn demonstrated more similar production patterns of acidic peptidase activities (Fig 2b).

Extracellular chitinase activities from all three isolates were estimated from the culture filtrates on both media (Fig 3). On wood-LNAS, low levels of chitinase production were already observed with peaking activities on the first weeks of cultivation for all isolates, and a later activity peak appeared on week 4 for the isolate Fp1243 (Fig 3a). On ME medium, the isolates Fp1181 and Fp1468 maintained low levels of chitinase activity until week 6 of cultivation, while the isolate Fp1243 demonstrated an increasing activity accumulation pattern until cultivation week 7, which then suddenly dropped within two weeks (cultivation weeks 7-9) to zero levels (Fig 3b).

Ergosterol content and fungal growth

Ergosterol analysis demonstrated the presence of active mycelial growth for all three isolates on both media during 12 weeks of cultivation (Table S1, Fig 4). On wood-LNAS, fungal growth was slower but continuous according to ergosterol concentrations in the extracted, fungal colonized wood (Fig 4a). However, after rapid mycelial ergosterol accumulation on ME medium until cultivation week 6, the ergosterol content declined by week 8 but showed increased ergosterol by the end of cultivation (on week 12) (Fig 4b). .

266 *Oxalic acid and extracellular pH*

267 On both (ME broth and wood-LNAS) cultivation media, the three *F. pinicola* isolates
268 accumulated substantial concentrations of oxalic acid over the cultivation period, resulting in
269 marked decrease in the culture fluid pH (Fig 5). Even on the buffered wood-LNAS medium
270 (pH 4.5), increasing acidity dropped the pH to 3.0-3.5 in five weeks of cultivation (Fig 5a).
271 Isolate Fp1468 showed the highest accumulation of 10 mM oxalic acid on cultivation week 8.
272 However, even more intense accumulation of oxalic acid was observed with all three isolates
273 when they were cultivated on ME medium, with oxalic acid concentrations reaching up to over
274 50 mM in cultures of the isolate Fp1181 on week 9 (Fig 5b). Accordingly, the culture fluid pH
275 values dropped dramatically on ME medium with a decrease in the pH from the initial value of
276 pH 5.0 to pH 1.5-2.0 already during the two first weeks of cultivation.

277 *Fe^{3+}-reducing activity*

278 Extracellular Fe^{3+}-reducing activity produced by the three *F. pinicola* isolates cultivated on ME
279 and wood-LNAS media, was analyzed from the culture fluid samples, and from ethyl acetate-
280 extracted concentrates at time points 0, 2, 8 and 12 weeks of cultivation (Fig 6). Trace
281 amounts of Fe^{3+}-reducing activity were detected in both media (ME liquid and wood-LNAS) at
282 the start of the cultivations (0 week). On both cultivation media, all isolates released variable
283 amounts of extracellular Fe^{3+}-reduction activity over the cultivation period, with no distinct
284 temporal tendency. However, the ethyl acetate-extracts demonstrated noticeable Fe^{3+}-
285 reducing activities, suggesting the constitutive presence of Fe^{3+}-reducing metabolites over the
286 cultivation period in wood-LNAS (Fig 6a) and ME (Fig 6b). Variability in the Fe^{3+}-reduction
287 activities may be a consequence of accumulation of fungal-produced oxalic acid in the culture
288 filtrates, which was tested by addition of reference oxalic acid (in Milli-Q water) in varying

289 concentrations (0-50 mM) to the ethyl acetate-extract of isolate Fp1468 from the 12 week ME
290 cultures (Fig S1).

291 *Fungal metabolites and Fe^{3+}-reducing activity*

292 In order to examine aromatic extracellular metabolites produced by the three *F. pinicola*
293 isolates on ME liquid and wood-LNAS media at the end-point of the cultivations (on week 12),
294 the ethyl acetate-extracted culture filtrates were further analyzed by UHPLC. Multiple potential
295 aromatic compounds were separated from the three isolates, as indicated by the sets of
296 peaks detected at 280 nm of absorbance (Fig 7, Table 2). HPLC chromatograms recorded at
297 260 nm (data not shown) indicated minor differences (mainly in the peak absorbance
298 intensities) in comparison to the peak profiles recorded at 280 nm.

299 All three isolates demonstrated similar profiles of extracellular aromatic compounds on both
300 media, with apparently a more versatile set of aromatic compounds secreted on ME medium
301 than was observed on wood-LNAS medium (Fig 7, Fig S2, Fig S3, Table 1). On ME medium,
302 *F. pinicola* isolates accumulated aromatic compounds (in 12 weeks) which were detected at
303 280 nm absorbance at retention times (RT) of 1.440, 2.031-2.042, 4.890, 10.012 and 11.221
304 min, and Fe^{3+}-reducing activity was detected in three of these peak fractions (peaks of RT
305 1.440, 2.031-2.042 and 10.012 min) (Table 1, Fig 7). On wood-LNAS medium, aromatic
306 compounds (as detected at 280 nm of absorbance) accumulated at RT 1.442, 2.799 and
307 10.125 min with Fe^{3+}-reducing activity detected for two of the fractions (peaks of RT 1.442
308 and 10.125 min) (Table 1, Fig 7). Similar pattern in generation of aromatic compounds was
309 observed with all three *F. pinicola* isolates on both media studied, with the same HPLC-
310 isolated compound fractions - demonstrating Fe^{3+}-reducing activity - shared between the

311 isolates (Table 1, Fig 6a,b,c,d; Fig S3-S4), which indicates on a fungal species-level and
312 constitutive specificity for production of bioactive metabolites.

313 **Discussion**

314 The biochemical mechanisms involved in the biodegradation of lignocelluloses and wood by
315 the brown-rot fungi is understood to be acquired as an alternative to the enzymatically and
316 genetically more expanded apparatus employed by the white-rot fungi (5-7, 18). Brown-rot
317 fungi are still efficient decomposers of lignocellulose, expressing strong oxidative
318 biochemistry (13-14, 24) and CAZy hydrolytic enzymes active on cellulose, hemicellulose and
319 pectin (1, 5-6, 18-20, 23, 36). The mechanisms, however, are not yet fully understood, and
320 variations of the biochemical processes and enzyme expression are expected to have
321 evolved in the separate (order-level) systematic lineages of Agaricomycetes brown-rot fungi.
322 Emerging evidence relays on a non-enzymatic free radical system generated *via* Fenton
323 chemistry to act on the lignocellulose structures leading to modification of hemicelluloses and
324 lignin units, and thus facilitating binding and infiltration of fungal secreted CAZys acting on
325 cellulose and hemicellulose polysaccharides (7, 9, 19).

326 According to structural localization study on decayed wood, it has been concluded that brown-
327 rot fungi depolymerizes hemicellulose prior to cellulose and degrade all polysaccharides in
328 the decay process (19-23, 38). This general order of polysaccharide decomposition is
329 supported by our study; all three *F. pinicola* isolates demonstrated extracellular hemicellulose-
330 targeted xylanase activities on the spruce wood-containing medium (wood-LNAS). Xylanase
331 activity was produced early on during the first week of cultivation, demonstrating a repeating
332 pattern of enzyme activity during the cultivation period of ten weeks. Surprisingly, isolate
333 Fp1243 expanded high xylanase activities during the same cultivation period. Repeating

pattern was also presented by cellulose-attacking endo-β-1,4-glucanase activities with some time-dependent differences between the three *F. pinicola* isolates. Likewise, time-dependent activity pattern in endo-β-1,4-glucanase activity has been observed for the other Polyporales brown-rot species *P. placenta* on solid wood (39). In the genomes of *P. placenta* and *F. pinicola*, multiple genes encoding CAZy GH classes responsible for xylanase and endo-β-1,4-glucanase activities are identified (6, 18), thus suggesting for expression of different genes producing divergent xylanases and endo-glucanase enzymes in the course of fungal hyphal growth and developing decay of wood. Previous studies on production of cellulose-attacking endo-β-1,4-glucanase activity by the brown-rot species *G. trabeum* proposed that the enzymes are expressed constitutively, and are thereby not subjected to accumulating glucose-influenced catabolic repression during lignocellulose decay (40-41). This is supported by our results on endo-glucanase activities produced by the three isolates of *F. pinicola*.

On the contrary to the cellulolytic endo-glucanase activity, brown-rot fungi are known to show low activities of β-glucosidase (4). This may be due to the β-glucosidase enzyme redundancy and differences in substrate specificities and cellular localization (5-7, 18), but also due to catabolic repression by glucose as end-product for the activity (19). Accordingly, the three *F. pinicola* isolates secreted overall low levels of β-glucosidase activity compared to the other CAZy activities analyzed, with a slightly fluctuating activity pattern observed during the course of the cultivation period. As observed for endo-glucanase activity, this pattern can be attributed to differential regulation of multiple β-glucosidase encoding genes expressed and subsequent isoenzymes secreted by the isolates.

Wood-decaying fungi secrete proteases as well as chitinases to acquire nitrogen as well as regeneration of the fungal mycelium during growth (42-43). The three isolates of *F. pinicola*

357 presented gradual increase in extracellular acidic peptidase activity over the cultivation
358 period, suggesting for a need of available organic nitrogen sources as the mycelia were
359 decomposing their lignocellulose substrates, particularly on the semi-solid wood-LNAS
360 medium. Proteolytic enzymes from *F. pinicola* have been reported to be active at acidic pH
361 (42), which is in accordance with our results on increasing acidic (pH 4.0 in assay) peptidase
362 activities detected for the three isolates during the cultivation period. Variable degrees of
363 extracellular chitinase activity were as well observed with the *F. pinicola* isolates, suggesting
364 intimate regulation of chitin biosynthesis and degradation as has been proposed for
365 filamentous fungi (43).

366 Accumulation of oxalic acid is a distinguishable characteristic of brown-rot fungi, and their
367 abilities for extracellular production of oxalic acid exceeds that of the evolutionarily near-
368 related white-rot fungi, increasing up to several tenfold concentrations (25, 44). We observed
369 increase in accumulation of oxalic acid during growth of the three *F. pinicola* isolates, with
370 even higher concentrations (over 50 mM) on the more rich ME medium. This may primarily be
371 a yield effect and consequence of the stronger mycelial growth on ME medium, as was
372 reflected by the substantially greater content of ergosterol determined in the mycelia from ME
373 cultivations than from the wood-LNAS cultures. Although significant secretion of oxalic acid is
374 common with *F. pinicola* (17), differences in the quantities of oxalic acid production were
375 observed between the three isolates in our study. Isolate Fp1468 presented oxalic acid
376 secretion more readily on the spruce-wood-LNAS-medium while the isolate Fp1181 was the
377 best producer of oxalic acid on the rich ME medium. Despite the differences in oxalic acid
378 production, final pH values of the culture fluids decreased to similar low levels by all three *F.*
379 *pinicola* isolates (around pH 3 on wood-LNAS, around pH 2 on ME medium). Increasing

extracellular acidity may, in turn, affect CAZy enzyme activities attacking hemicelluloses and cellulose, as well as protein-degrading enzymes (4, 29).

Oxalic acid and low pH have been implicated in Fenton-chemistry based free radical-generation by brown-rot fungi (9, 16-17, 44). *In vitro* experiments have demonstrated that oxalic acid as soluble oxalate anions may easily chelate metal cations, like ferric iron from wood, in the low pH environment in the vicinity of fungal hyphae within the lumen area, thus preventing reduction of iron species and subsequent generation of hydroxyl radicals via Fenton reaction (16-17, 24). In this study, we observed that the three *F. pinicola* isolates demonstrated Fe^{3+}-reducing activities on both substrate media and over the long cultivation period. The Fe^{3+}-reducing activity was found to be constitutively secreted at all time analyzed during the cultivation period, as shown by the activity measurements from ethyl acetate extract, suggesting for constant presence of extracellular metabolites with Fe^{3+}-reduction potential. The Fe^{3+}-reducing activity observed in the ethyl acetate extracted culture filtrates also suggest the possible inhibitory effect of oxalic acid on Fe^{3+}-reduction. This conclusion was further supported by the observed decline in Fe^{3+}-reducing activity of extracted culture filtrate of the isolate Fp1468 by addition of increasing molar concentrations of oxalic acid. Unlike in a recent study on distinguishing fungal hyphae-generated biochemical gradients and decay phases of brown-rot on wood (36), our goal here was to initially analyze the enzymatic and non-enzymatic players in the lignocellulose-degradative system of *F. pinicola*. Our next aim is to identify these processes on solid-wood cultivations, in order to aid in comparison of the proposed several-stage and “staggered” decay mechanism of *P. placenta* (36) to the decay events and physiology of *F. pinicola*, and in general for brown-rot Polyporales fungi.

Basidiomycota hyphae-secreted metabolites have been reported to possess Fe^{3+}-reducing activity while growing on wood and lignocellulose-supplemented media, or on soil extract, and these biocompounds have been as well implicated to play a role in Fenton chemistry (3, 5, 34). In this study, we managed to identify a number of aromatic compound fractions secreted by the three isolates of *F. pinicola*, with a few accumulating compounds showing Fe^{3+}-reducing activity. A number of these potential iron reducing-compounds were produced by all three isolates thus suggesting for both (i) a constitutive biosynthetic pathway for production of specific bioactive secondary metabolites (as was observed on ME medium), and (ii) similarities in bioconversion of spruce-wood derived soluble aromatic compounds (as was observed on wood-LNAS medium). Thus, species-specific Fenton-chemistry and wood decay mechanism may be tentatively formulated for *F. pinicola*. Identification of these extracellular bioactive compounds is our next step in order to elucidate, which roles they may play in Fenton chemistry and brown-rot decay generated by the fungus.

Our study confirmed that the three forest wild-type isolates of *F. pinicola* demonstrated extracellular enzyme activities to degrade lignocellulose polysaccharides, as well as proteolytic substrates, with potential for oxidative decay via Fenton chemistry, supported by strong secretion of oxalic acid and production of aromatic compounds. A few of the compounds were able to cause reduction of ferric iron species to ferrous ions. Production of the extracellular bioactive compounds with Fe^{3+}-reduction potential together with CAZyme activities suggests for a complex and fine-tuned mechanism that can be genetically and biochemically regulated by the fungus. Further analyses on the fungal proteome and functional genomics on wood, and characterization of the reducing-activity metabolites are necessary steps to uncover the brown-rot decay system of *F. pinicola*.

Acknowledgements

The study was supported by funding from the Academy of Finland (project grant 285676 Fungcolife, to TL), and the University of Helsinki, Faculty of Agriculture and Forestry, Department of Food and Environmental Sciences. Nina Sipari and Hans Mattila are thanked for the advice in HPLC analytics.

References:

1. **Hori C, Gaskell J, Igarashi K, Samejima M, Hibbett D, Henrissat B, Cullen D**. 2013. Genomewide analysis of polysaccharides degrading enzymes in 11 white- and brown-rot Polyporales provides insight into mechanisms of wood decay. Mycologia **105**:1412-1427.
2. **Binder M, Justo A, Riley R, Salamov A, Lopez-Giraldez F, Sjökqvist E, Copeland A, Foster B, Sun H, Larsson E, Larsson K-H, Townsend J, Grigoriev I, Hibbett D**. 2013. Phylogenetic and phylogenomic overview of the Polyporales. Mycologia **105**:1350-1373.
3. **Eriksson KEL, Blanchette RA, Ander P**. 1990. Microbial and enzymatic degradation of wood and wood components. Springer, Berlin, pp 1–72. ISBN: 978-3-642-46687-8.
4. **Baldrian P, Valaskova V.** 2008. Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol Rev **32:**501-521.
5. **Eastwood DC, Floudas D, Binder M, Majcherczyk A, Schneider P, Aerts A, Asiegbu FO, Baker SE, Barry K, Bendiksby M, Blumentritt M, Coutinho PM, Cullen D, de Vries RP, Gathman A, Goodell B, Henrissat B, Ihrmark K, Kauserud H, Kohler A, LaButti K, Lapidus A, Lavin JL, Lee YH, Lindquist E, Lilly W, Lucas S, Morin E, Murat C, Oguiza JA, Park J, Pisabarro AG, Riley R, Rosling A,**

Salamov A, Schmidt O, Schmutz J, Skrede I, Stenlid J, Wiebenga A, Xie XF, Kües U, Hibbett DS, Hoffmeister D, Hogberg N, Martin F, Grigoriev IV, Watkinson SC. 2011. The plant cell wall-decomposing machinery underlies the functional diversity of forest fungi. Science **333**:762–765.

6. **Floudas D, Binder M, Riley R, Barry K, Blanchette RA, Henrissat B, Martinez AT, Otillar R, Spatafora JW, Yadav JS, Aerts A, Benoit I, Boyd A, Carlson A, Copeland A, Coutinho PM, de Vries RP, Ferreira P, Findley K, Foster B, Gaskell J, Glotzer D, Gorecki P, Heitman J, Hesse C, Hori C, Igarashi K, Jurgens JA, Kallen N, Kersten P, Kohler A, Kues U, Kumar TK, Kuo A, LaButti K, Larrondo LF, Lindquist E, Ling A, Lombard V, Lucas S, Lundell T, Martin R, McLaughlin DJ, Morgenstern I, Morin E, Murat C, Nagy LG, Nolan M, Ohm RA, Patyshakuliyeva A, Rokas A, Ruiz-Dueñas FJ, Sabat G, Salamov A, Samejima M, Schmutz J, Slot JC, John F-St., Stenlid J, Sun H, Sun S, Syed K, Tsang A, Wiebenga A, Young D, Pisabarro A, Eastwood D, Martin F, Cullen D, Grigoriev IV, Hibbett DS.** 2012. The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science **336:**1715–1719.
7. **Lundell TK, Mäkelä MR, de Vries RP, Hildén KS**. 2014. Genomics, lifestyles and future prospects of wood-decay and litter-decomposing Basidiomycota. *In*: Martin F (ed.) The Fungi. Adv Bot Res **70**: 329-370.
8. **Schwarze FWMR**. 2007. Wood decay under the microscope. Fungal Biol Rev **21**:133–170.
9. **Arantes V, Jellison J, Goodell B.** 2012. Peculiarities of brown-rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass. Appl Microbiol Biotechnol **94**:323-338.

10. **Högberg N, Holdenrieder O, Stenlid J.** 1999. Population structure of the wood decay fungus *Fomitopsis pinicola*. Heredity **83**:354-360.
11. **Hammel KE, Kapich AN, Jensen KA Jr, Ryan ZC**. 2002. Reactive oxygen species as agents of wood decay by fungi. Enzyme Microb Technol **30**:445-453.
12. **Koenigs JW**. 1974. Hydrogen peroxide and iron: a proposed system for decomposition of wood by brown-rot basidiomycetes. Wood Fiber **6**:66–79.
13. **Kerem Z, Jensen KA, Hammel KE**. 1999. Biodegradative mechanism of the brown rot basidiomycete *Gloeophyllum trabeum*: evidence for an extracellular hydroquinone-driven Fenton reaction. FEBS Lett **446**:49–54.
14. **Jensen KA Jr, Houtman CJ, Ryan ZC, Hammel KE.** 2001. Pathways for extracellular Fenton chemistry in the brown rot basidiomycete *Gloeophyllum trabeum*. Appl Environ Microbiol **67:**2705–2711.
15. **Hyde SM, Wood PM**. 1997. A mechanism for production of hydroxyl radicals by the brown-rot fungus *Coniophora puteana*: Fe (II) reduction by the cellobiose dehydrogenase and Fe (II) oxidation at a distance from the hyphae. Microbiol **143**:259-266.
16. **Arantes V, Qian Y, Milagres AMF, Jellison J, Goodell B**. 2009. Effect of pH and oxalic acid on the reduction of Fe^{3+} by a biomimetic chelator and on Fe^{3+} desorption/adsorption onto wood: implications for brown rot decay. Int Biodeterior Biodegradation **63**:478-483.
17. **Schilling JS, Jellison J.** 2005. Oxalate regulation by two brown rot fungi decaying oxalate-amended and non-amended wood. Holzforschung **59:**681-688.

18. **Martinez D, Challacombe J, Morgenstern I, Hibbett D, Schmoll M, Kubicek CP, Ferreira P, Ruiz-Duenas FJ, Martinez AT, Kersten P, Hammel KE, Vanden Wymelenberg A, Gaskell J, Lindquist E, Sabat G, Bondurant SS, Larrondo LF, Canessa P, Vicuna R, Yadav J, Doddapaneni H, Subramanian V, Pisabarro AG, Lavin JL, Oguiza JA, Master E, Henrissat B, Coutinho PM, Harris P, Magnuson JK, Baker SE, Bruno K, Kenealy W, Hoegger PJ, Kues U, Ramaiya P, Lucas S, Salamov A, Shapiro H, Tu H, Chee CL, Misra M, Xie G, Teter S, Yaver D, James T, Mokrejs M, Pospisek M, Grigoriev IV, Brettin T, Rokhsar D, Berka R, Cullen D.** 2009. Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. Proc Natl Acad Sci USA **106**:1954–1959.

19. **Valáskova V, Baldrian P**. 2006. Degradation of cellulose and hemicelluloses by the brown rot fungus *Piptoporus betulinus*–production of extracellular enzymes and characterization of the major cellulases. Microbiol **152**:3613-3622.

20. **Milagres AMF, Sales RM**. 2001. Evaluating the basidiomycetes *Poria medula-panis* and *Wolfiporia cocos* for xylanase production. Enzyme Microb Technol **28**:522-526.

21. **Highley TL**. 1987. Effect of carbohydrate and nitrogen on hydrogen peroxide formation by wood decay fungi in solid medium. FEMS Microbiol Lett **48**:373-377.

22. **Highley TL, Illman BL**. 1991. Progress in understanding how brown-rot fungi degrade cellulose. Biodeterior Abstracts **5**:231-244.

23. **Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS**. 2005. Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol **67**:577-591.

517 **24. Goodell B, Jellison J, Liu J, Daniel G, Paszczynski A, Fekete F, Krishnamurthy S,**
518 **Jun L, Xu G**. 1997. Low molecular weight chelators and phenolic compounds isolated
519 from wood decay fungi and their role in the fungal biodegradation of wood. J
520 Biotechnol **53**:133-162.
521 **25. Mali T, Kuuskeri J, Shah F, Lundell T.** 2017. Interactions affect hyphal growth and
522 enzyme profiles in combinations of coniferous wood-decaying fungi of Agaricomycetes.
523 PLOS ONE, accepted on September 15, 2017.
524 **26. Kuuskeri J, Mäkela MR, Isotalo J, Oksanen I, Lundell T.** 2015. Lignocellulose-
525 converting enzyme activity profiles correlate with molecular systematics and phylogeny
526 grouping in the incoherent genus *Phlebia* (Polyporales, Basidiomycota). BMC Microbiol
527 **15**:217.
528 **27. Rytioja J, Hildén K, Hatakka A, Mäkelä MR**. 2014. Transcriptional analysis of
529 selected cellulose-acting enzymes encoding genes of the white-rot fungus *Dichomitus*
530 *squalens* on spruce wood and microcrystalline cellulose. Fungal Genet Biol **72**:91-98.
531 **28. Bailey MJ, Biely P, Poutanen K**. 1992. Interlaboratory testing of methods for assay of
532 xylanase activity. J. Biotechnol **23**:257-270.
533 **29. Shah F, Rineau F, Canbäck B, Johansson T, Tunlid A.** 2013. The molecular
534 components of the extracellular protein-degradation pathways of the ectomycorrhizal
535 fungus *Paxillus involutus*. New Phytol **200**:875-887.
536 **30. Pritsch K, Courty PE, , Churin J-L , Cloutier-Hurteau B, Ali MA, Damon C,**
537 **Duchemin M, Egli S, Ernst J, Fraissinet-Tachet L, Kuhar F, Legname E,**
538 **Marmeisse R, Müller A, Nikolova P, Peter M, Plassard C, Richard F, Schloter M,**
539 **Selosse M-A, Franc A, Garbaye J**. 2011. Optimized assay and storage conditions for
540 enzyme activity profiling of ectomycorrhizae. Mycorrhiza **21**:589-600.

541 **31. Mattila H, Kuuskeri J, Lundell T**. 2017. Single-step, single-organism bioethanol
542 production and bioconversion of lignocellulose waste materials by phlebioid fungal
543 species. Bioresource Technol **225**:254-261.
544 **32. Niemenmaa O, Galkin S, Hatakka A**. 2008. Ergosterol contents of some wood-rotting
545 basiodiomycete fungi grown in liquid and solid culture conditions. Int Biodeterior
546 Biodegradation **62**:125-134.
547 **33. Frostegård A, Bååth E**. 1996. The use of phospholipid fatty acid analysis to estimate
548 bacterial and fungal biomass in soil. Biol Fertil Soils **22**:59-65.
549 **34. Shah F, Schwenk D, Nicolas C, Persson P, Hoffmeister D, Tunlid A.** 2015. Involutin
550 is a Fe^{3+} reductant secreted by the ectomycorrhizal fungus *Paxillus involutus* during
551 Fenton-based decomposition of organic matter. Appl Environ Microbiol **81**:8427-8433.
552 **35. Ruiz-Dueñas FJ, Lundell T, Floudas D, Nagy LG, Barrasa JM, Hibbett DS,**
553 **Martinez AT**. 2013. Lignin-degrading peroxidases in Polyporales: and evolutionary
554 survey based on 10 sequenced genomes. Mycologia **105**:1428-1444.
555 **36. Zhang J, Presley GN, Hammel KE, Ryu J-S, Menke JR, Figueroa M, Hu D, Orr G,**
556 **Schilling JS**. 2016. Localizing gene regulation reveals a staggered wood decay
557 mechanism for the brown rot fungus *Postia placenta*. Proc Natl Acad Sci USA
558 **27**:10968-10973.
559 **37. Schilling JS, Duncan SM, Presley GN, Filley TR, Jurgens JA, Blanchette RA.**
560 2013. Colocalizing incipient reactions in wood degraded by the brown rot fungus *Postia*
561 *placenta.* Int Biodeterior Biodegradation **83**:56-62.
562 **38. Blanchette RA, Abad AR**. 1988. Ultrastructural localization of hemicellulose in birch
563 wood (*Betula papyfera*) decayed by brown and white-rot fungi. Holzforschung **42**:393-
564 398.

565 **39. Duncan SM, Schilling JS**. 2010. Carbohydrate-hydrolyzing enzyme ratios during
566 fungal degradation of woody and non-woody lignocellulose substrates. Enzyme Microb
567 Technol **47**:363-371.
568 **40. Cotoras M, Agosin E**. 1992. Regulatory aspects of endoglucanase production by the
569 brown-rot fungus *Gloeophyllum trabeum*. Exp Mycol **16**:253–260.
570 **41. Highley TI**. 1973. Influence of carbon source on cellulase activity of white-rot and
571 brown-rot fungi. Wood Fiber **5**:50-58.
572 **42. Kudryavtseva OA, Dunaevsky YE, Kamzolkina OV, Belozersky MA**. 2008. Fungal
573 proteolytic enzymes: features of the extracellular proteases of xylotrophic
574 basidiomycetes. Microbiol **77**:643-653.
575 **43. Langner T, Göhre V**. 2016. Fungal chitinases: function, regulation, and potential roles
576 in plant/pathogen interactions. Curr Genet **62**:243-254.
577 **44. Mäkelä MR, Hildén K, Lundell TK**. 2010. Oxalate decarboxylase: biotechnological
578 update and prevalence of the enzyme in filamentous fungi. Appl Microbiol Biotechnol
579 **87**:801-814.

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583

584 **Figure legends**

585 **Fig 1.** Extracellular enzyme activities of *Fomitopsis pinicola* against hemicellulose and
586 cellulose. Endo-β-1,4-xylanase (a), endo-β-1,4-glucanase (b), and β-glucosidase (c) activities
587 were detected from wood-LNAS cultures during 10 weeks of cultivation. *F. pinicola* isolates
588 1181 (dotted line, closed square), 1243 (long dash dot, closed triangle) and 1468 (continuous
589 line, closed circle). Bars indicate ±SE (*n*=5, biological replicates). In some cases, the error bar
590 is not visible due to low deviation values.

591 **Fig 2.** Extracellular acidic protease activity of *F. pinicola* isolates on (a) wood-LNAS, and (b)
592 ME medium, during 10 weeks of cultivation. Isolates 1181 (dotted line, closed square), 1243
593 (long dash dot, closed triangle) and 1468 (continuous line, closed circle). Peptidase activity is
594 represented as activity generated by amount of Pepsin equivalent in mg/L of culture filtrate.
595 Bars indicate ±SE (*n*=5, biological replicates). In some cases, the error bar is not visible due
596 to low deviation values.

597 **Fig 3.** Extracellular chitinase activity of *F. pinicola* isolates on (a) wood-LNAS, and (b) ME
598 medium, during 10 weeks of cultivation. Isolates 1181 (dotted line, closed square), 1243 (long
599 dash dot, closed triangle) and 1468 (continuous line, closed circle). Chitinase activity is
600 represented as the amount of 4-methylumbelliferone (MU) released per L of culture filtrate.
601 Bars indicate ±SE (*n*=5, biological replicates). In some cases, the error bar is not visible due
602 to low deviation values.

603 **Fig 4.** Ergosterol content of the *F. pinicola* isolates on (a) wood-LNAS, and (b) ME medium,
604 during 12 weeks of cultivation. Ergosterol content is estimated per g dry weight fungal

605 colonized wood (wood-LNAS), and dry weight fungal mycelia (ME). Bars indicate ±SE (*n*=3,
606 biological replicates). In some cases, the error bar is not visible due to low deviation values.

607 **Fig 5.** Accumulation of secreted oxalic acid and decrease of culture filtrates pH during
608 cultivations of the three *F. pinicola* isolates on (a) wood-LNAS, and (b) ME medium over the
609 10-week cultivation period. Oxalic acid concentrations are represented as columns, and pH
610 values are indicated as line graphs. Single legend box identifies oxalic acid production and pH
611 for both media. Bars indicate ±SE (*n*=5, biological replicates). In some cases, the error bar is
612 not visible due to low deviation values.

613 **Fig 6.** Fe^{2+}-generation by the culture filtrate (CF) and ethyl acetate-extracted culture filtrate
614 (ET) obtained from (a) wood-LNAS, and (b) ME media. Activities for time points of 2, 8 and 12
615 weeks of cultivation are presented for all three *F. pinicola* isolates, and are normalized against
616 Fe^{2+}-generation detected in the un-inoculated sterile medium (control samples derived on day
617 0 of cultivations). Fe^{2+} generated are represented per liter (L) of culture filtrate for both CF
618 and ET. Bars indicate ±SE (*n*=3, biological replicates). In some cases, the error bar is not
619 visible due to low deviation values.

620 **Fig 7**. Aromatic compound profiles in cultures of the *F. pinicola* isolate 1468 cultivated on
621 wood-LNAS and ME media. HPLC chromatograms of ethyl acetate-extracted culture filtrates
622 were derived at time points of (a) 0, initial medium-dissolved compound profile of wood-LNAS
623 substrate at the beginning of cultivation; (b) after 12 weeks of cultivation on wood-LNAS; (c)
624 0, initial medium-dissolved compound profile of ME substrate at the beginning of cultivation;
625 and (d) after 12 weeks of cultivation on ME medium. Aromatic compound peaks predicted to
626 be generated as either secretion products or by bio-conversion of medium-existing aromatic

627 and organic molecules, are indicated with their retention times. For peaks with Fe^{3+}-reducing
628 activities, see Table 1.

629

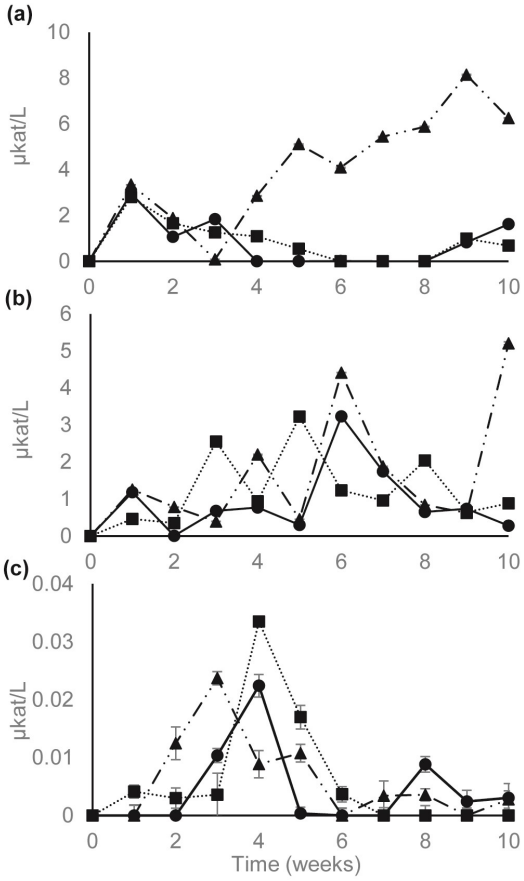

Fig. 1

630

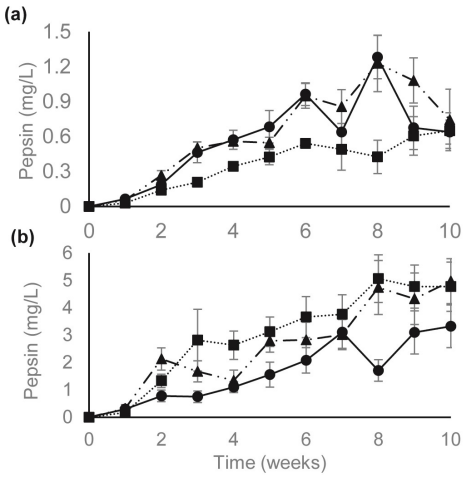
(a)
Pepsin (mg/L)
1.5
1.2
0.9
0.6
0.3
0
0
2
4
6
8
10
(b)
Pepsin (mg/L)
6
5
4
3
2
1
0
0
2
4
6
8
10
Time (weeks)

Fig. 2

631

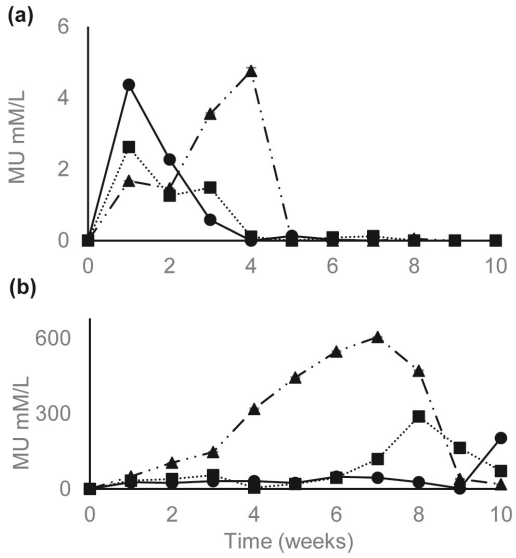

Fig. 3

632

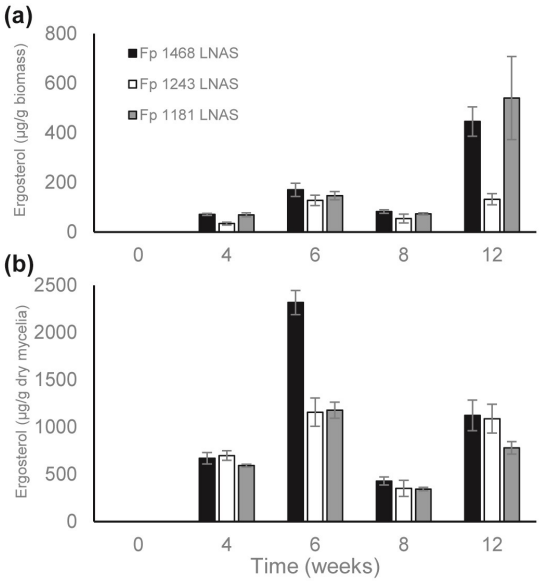
(a)
800
600
400
200
0
Ergosterol (µg/g biomass)
Fp 1468 LNAS
Fp 1243 LNAS
Fp 1181 LNAS
0
4
6
8
12
(b)
2500
2000
1500
1000
500
0
Ergosterol (µg/g dry mycelia)
0
4
6
8
12
Time (weeks)

Fig. 4

633

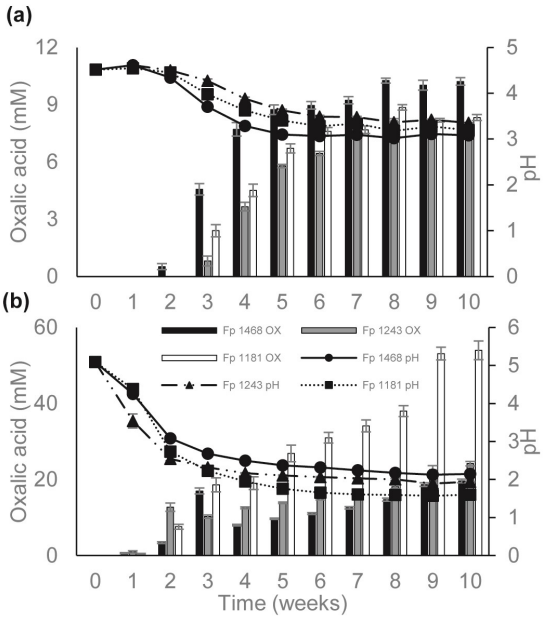
(a)
Oxalic acid (mM)
pH
(b)
Fp 1468 OX
Fp 1243 OX
Fp 1181 OX
Fp 1468 pH
Fp 1243 pH
Fp 1181 pH
Oxalic acid (mM)
pH
Time (weeks)

Fig. 5

634

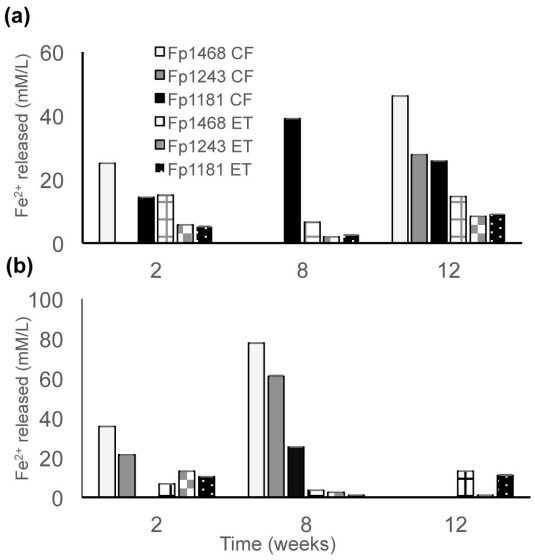
(a)
Fp1468 CF
Fp1243 CF
Fp1181 CF
Fp1468 ET
Fp1243 ET
Fp1181 ET
Fe2+ released (mM/L)
60
40
20
0
2
8
12
(b)
Fe2+ released (mM/L)
100
80
60
40
20
0
2
8
12
Time (weeks)

Fig. 6

635

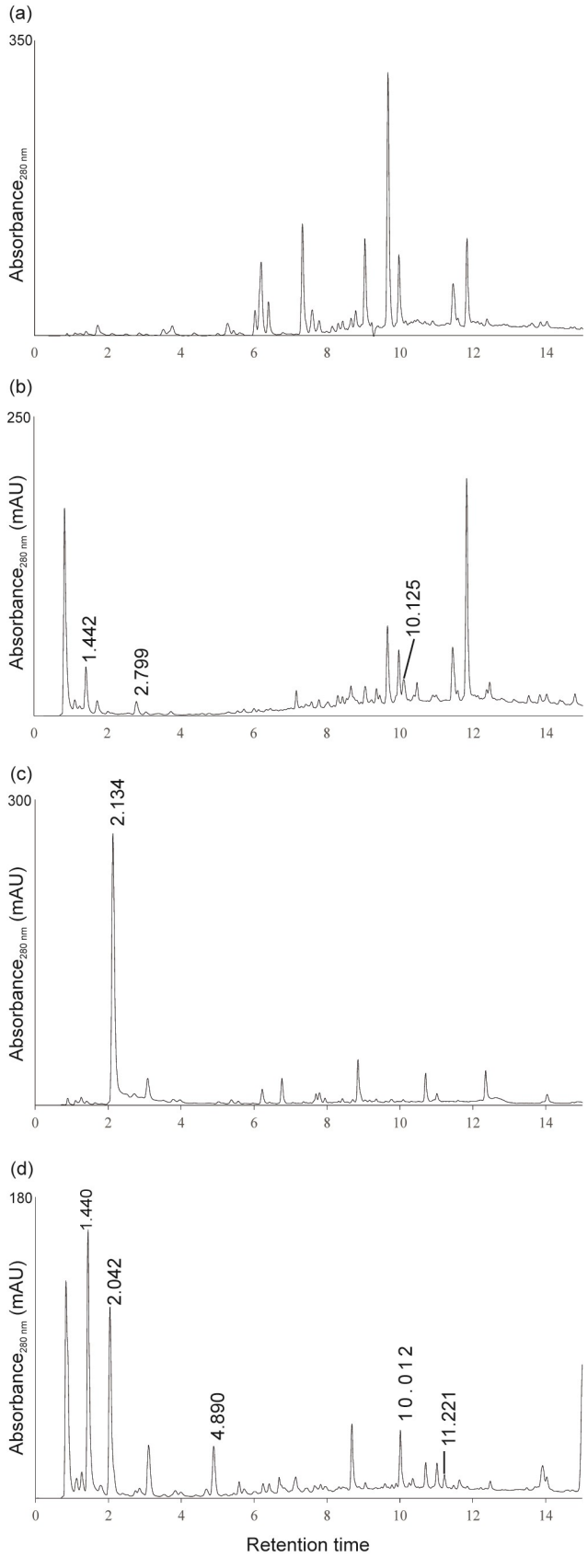
(a)
350
Absorbance280 nm
0
2
4
6
8
10
12
14
(b)
250
Absorbance280 nm (mAU)
1.442
2.799
10.125
(c)
300
2.134
(d)
180
1.440
2.042
4.890
10.012
11.221
Retention time

Fig. 7

636

637 **Tables**

638

639 **Table 1**. Aromatic compound peaks analyzed and collected by HPLC from the ethyl-acetate
640 extracted culture filtrate samples of the three isolates of *F. pinicola* cultivated on wood-LNAS
641 and ME media. Fe^{3+}-reducing activity of released peaks were performed using ferrozine
642 assay. RT: retention time (min).

Fungal isolate (Fp)	Aromatic compound peaks in the cultures (RT, A 280 nm)		Fe^{3+}-reducing peaks (RT, A 280 nm)	
	wood-LNAS	ME	wood-LNAS	ME
1181	**1.418**, 2.799, **10.119**	**1.440**, **2.031**, 2.377, 4.855, **9.999**	1.418, 10.119	1.440, 2.031, 9.999
1243	**1.420**, 2.796, **10.127**	1.822, **2.031**, **9.999**	1.420, 10.127	2.031, 9.999
1468	**1.442**, 2.799, **10.125**	**1.440**, **2.042**, 4.890, **10.012**,11.221	1.442, 10.125	1.440, 2.042, 10.012

643

644

645

646 ***Applied and Environmental Microbiology*** **Supporting Information**

647 Article title: **Profiles of enzyme activities, oxalic acid productions and Fe^{3+}-reducing metabolite**
648 **secreted by the Polypore *Fomitopsis pinicola*.**

649

650 Authors: Firoz Shah, Tuulia Mali, Taina Lundell

651

652

653 The following Supporting Information is available for this article:

654 **Table S1.** Ergosterol content for *F.pinicola* isolates cultivated on malt extract broth (ME) and

655 wood-LNAS. Three parallel cultivation were used (±SE). Ergosterol content are expressed as

656 micrograms per gram of dry weight mycelia (ME) and dry weight biomass of colonized wood.

657

658 **Fig S1.** Effect of oxalic acid on iron-reducing activity (Fe^{3+} to Fe^{2+}) of ethyl acetate-extracted

659 culture filtrate of *F. pinicola* 1468 after 12 weeks of cultivation on ME medium. Bars indicate

660 ±SE (technical replicate, *n*=3)

661 **Fig S2.** Aromatic metabolite profiles produced by *F. pinicola* isolates 1181 and 1243 after 12

662 weeks of cultivation on wood-LNAS medium. HPLC chromatograms.

663 **Fig S3.** Aromatic metabolite profiles produced by *F. pinicola* isolates 1181 and 1243 after 12

664 weeks of cultivation on ME medium. HPLC chromatograms.

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Table S1. Ergosterol content for *F.pinicola* isolates cultivated on malt extract broth (ME) and wood-LNAS. Three parallel cultivation were used (±SE). Ergosterol content are expressed as micrograms per gram of dry weight mycelia (ME) and dry weight biomass of colonized wood.

	Ergosterol (µg/g ±SE dry weight mycelia for ME or wood biomass for wood-LNAS)					
	Fp1181		Fp1243		Fp1468	
Growth period (w)	ME	wood-LNAS	ME	wood-LNAS	ME	wood-LNAS
0	0	0	0	0	0	0
4	592 ±14	69 ±7	698 ±51	33 ±5	669 ±60	70 ±4
6	1178 ±84	146 ±16	1157 ±148	127 ±21	2317 ±127	170 ±26
8	344 ±18	73 ±4	351 ±84	54 ±18	429 ±43	82 ±7
12	780 ±66	540 ±167	1088 ±152	132 ±22	1124 ±162	445 ±59

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690 **Fig S1.** Effect of oxalic acid on iron-reducing activity (Fe^{3+} to Fe^{2+}) of ethyl acetate-extracted

691 culture filtrate of *F. pinicola*1468 after 12 weeks of cultivation on ME medium. Bars indicate

692 ±SE (technical replicate, *n*=3)

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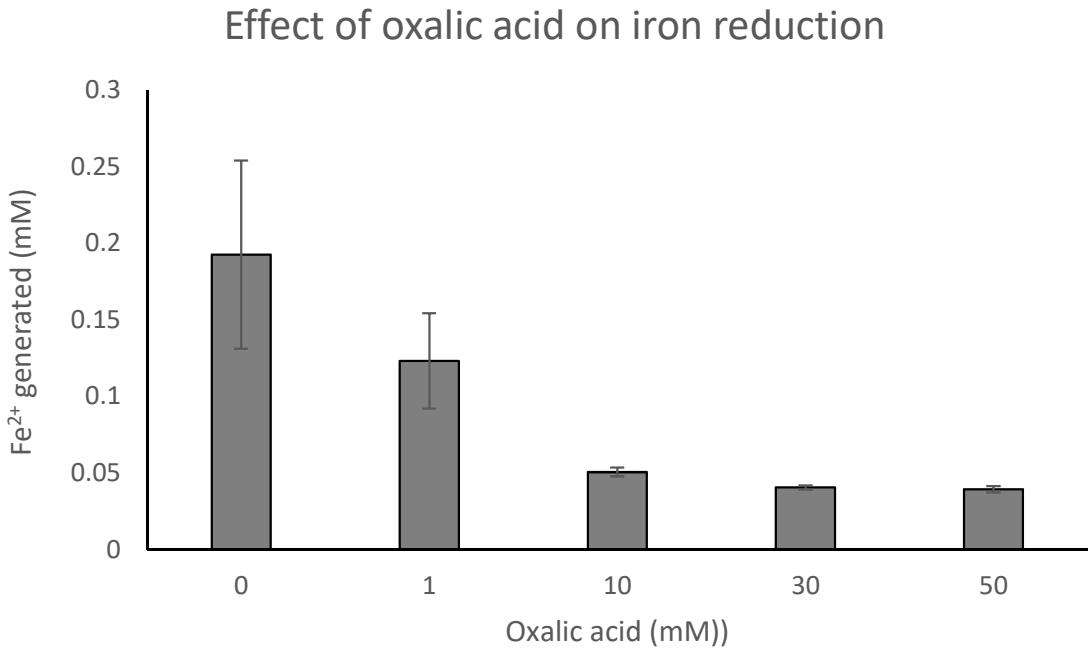

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Fig S2. Aromatic metabolite profiles produced by *F. pinicola* isolates 1181 and 1243 after 12 weeks of cultivation on wood-LNAS medium. HPLC chromatograms.

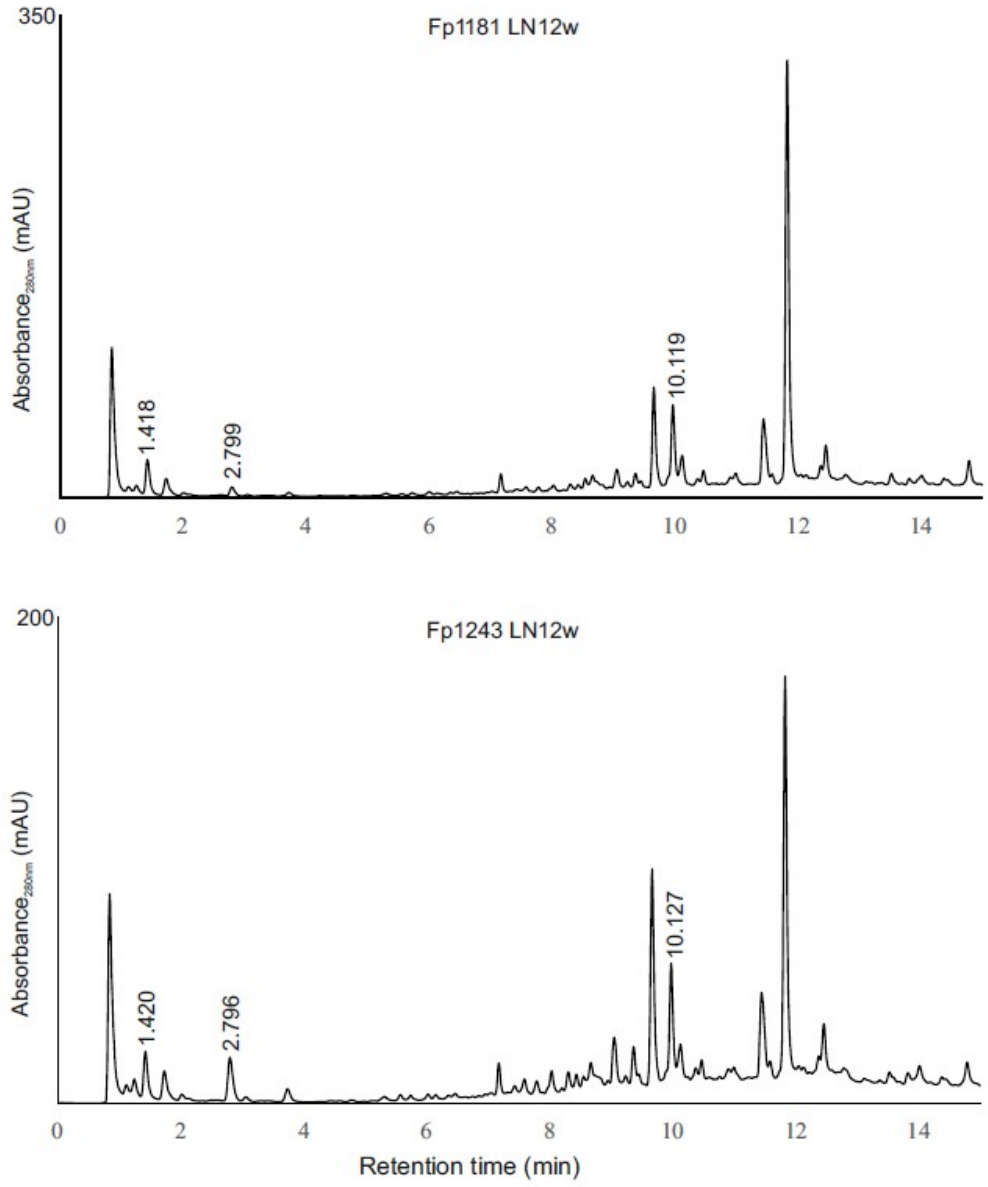

Fig S3. Aromatic metabolite profiles produced by *F. pinicola* isolates 1181 and 1243 after 12 weeks of cultivation on ME medium. HPLC chromatograms.